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Dietary rice bran supplementation prevents *Salmonella* colonization differentially across varieties and by priming intestinal immunity

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ABSTRACT

The global burden of enteric dysfunction and diarrhoeal disease remains a formidable problem that requires novel interventions. This study investigated the immune-modulatory capacity of bran across rice varieties with phytochemical differences. 129SvEvTac mice were fed a 10% rice bran or control diet followed by infection with *Salmonella enterica*. Faecal shedding titres were quantified and flow cytometry was used to investigate intestinal immunity. The largest protection against *Salmonella* colonization was observed with IAC600 variety. Reduced faecal shedding correlated with increased levels of boron, soluble fibre, vitamin E isomers, and fatty acids. IAC600 and Red Wells rice bran modulated small intestinal neutrophils, macrophages, interdigitating dendritic cells, $CD8^+$, γδ, and regulatory T cells, as well as $CD8^+$ and γδ T cells in the mesenteric lymph nodes. Rice bran is a promising functional food and merits evaluation for the prevention of *Salmonella* colonization and regulation of intestinal immunity in people.

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1. Introduction

Enteric disease remains a major global health problem, with diarrhoeal disease remaining a top killer of children in developing nations (Levine, Kotloff, Nataro, & Muhsen, 2012). *Salmonella* spp. are one of a diverse set of enteric pathogens that contribute to diarrhoeal disease in both developed and developing regions of the world with similar incidence rates of 240 and 540 per 100,000 in developed nations of W. Europe and N. America and 320 and 1440 per 100,000 in developing nations of Africa and Southeast Asia, respectively (Kotloff et al., 2013; Majowicz et al., 2010; Petri et al., 2008). *Salmonella* enteritis is a major cause of malnutrition and growth retardation, two major complications of diarrhoeal disease (Petri et al., 2008). Protection against *Salmonella* infection involves coordination of innate and adaptive immune responses involving helper $CD4^+$ T cells, and to a lesser extent cytotoxic $CD8^+$ T cells and antibody production (Griffin & McSorley, 2011; Salerno-Goncalves, Fernandez-Vina, Lewinsohn, & Sztein, 2004). Antibiotic treatment of non-typhoidal *Salmonella* infection has a minimal impact on diarrhoea, and while new vaccines are improving protection, there remains a strong need for novel therapies (Anwar et al., 2014; Onwuezobe, Oshun, & Odigwe, 2012). Nutritional therapies that have shown promise for controlling salmonellosis include dietary fructooligosaccharides, short chain fatty acids (SCFA), barley, and rice hull extract in animal models (Bovee-Oudenhoven, ten Bruggencate, Lettink-Wissink, & van der Meer, 2003; Boyen et al., 2008; Kim, Kang, Park, Nam, & Friedman, 2012; Michiels et al., 2012; Pieper et al., 2012) as well as green banana and pectin, and dietary lactic acid fermented cereal gruel in humans (Kingamkono, Sjogren, & Svanberg, 1999; Rabbani et al., 2001). Though multiple interventions have been tested, little is known about immune responses following nutritional intervention. Interestingly, studies in weaning piglets demonstrated that protection following arginine supplementation resulted in reduced serum C reactive protein as well as (systemic or local) tissue expression of inflammatory biomarkers Myd88, TLR4, TLR5, NFκB and TNF-α following systemic *Salmonella* infection (Y. Chen et al., 2012). Recent studies from our laboratory have highlighted the potential of bran from the rice variety, "Neptune," to reduce *Salmonella* colonization using *in-vitro* and mouse models. In this case, protection is associated with increased native gut *Lactobacillus* numbers and reduced production of systemic pro-inflammatory cytokine production (Henderson, Kumar, Barnett, Dow, & Ryan, 2012; Kumar et al., 2012).

Rice is a global staple food crop and is thought to account for one fifth of the calories consumed worldwide (Sharif, Butt, Anjum, & Khan, 2014). Therefore, rice bran is presently available in most regions of the world, but is typically discarded as a by-product of rice polishing. Rice bran contains a number of bioactive components and has been shown to have a beneficial impact on numerous diseases including colon cancer, heart disease, and *Salmonella* infections (Forster et al., 2013; Henderson, Ollila et al., 2012; Jariwalla, 2001; Kim, Park, Lee, Nam, & Friedman, 2013, 2014; Kumar et al., 2012; Kuriyan, Gopinath, Vaz, & Kurpad, 2005). Additional studies have shown other benefits including antioxidant, antitumour, anti-atherosclerosis, diabetes control, and anti-allergy potential (Cheng et al., 2010; Forster et al., 2013; Goufo & Trindade, 2014; Henderson, Ollila et al., 2012; Nicolosi, Ausman, & Hegsted, 1991; Oka et al., 2010). A number of genetically and agronomically diverse rice varieties have been developed resulting in different grain sizes, aromas and nutritional qualities (Marr, Batten, & Blakeney, 1995; Rutger et al., 2004; Saenchai, Prom-u-thai, Jamjod, Dell, & Rerkasem, 2012; Schaeffer, Sharpe, & Dudley, 1994), and protection against plant diseases and pests (Fitzgerald, McCouch, & Hall, 2009; Huang et al., 2010; Khush, 1997; Ni, Colowit, & Mackill, 2002; Zhu et al., 2000). Varietal differences in bioactivity of rice bran have been observed in anti-tumour capacity (M. H. Chen, Choi, Kozukue, Kim, & Friedman, 2012; Forster et al., 2013). In the context of *Salmonella*, evidence supporting the importance of varietal differences has also been demonstrated. For instance, carbohydrate levels amongst barley varieties impacted *Salmonella* shedding from pigs, and rice bran extracts from Sung-Yod and Jasmine rice varieties had differential *in-vitro* antimicrobial capacity against *Salmonella* (Kondo, Teongtip, Srichana, & Itharat, 2011; Pieper et al., 2012).

The ability of rice bran to influence immunity has also been demonstrated. Feruloylated oligosaccharides from rice bran activated bone marrow derived dendritic cells by increasing CD40, CD86 and MHC-II expression in a TLR-4 and TLR-2 dependent fashion (Lin et al., 2014). In addition, rice bran oil consumption enhances systemic B cell proliferation and Th1 cytokines while suppressing Th2 cytokines and IgE (Sierra et al., 2005). Furthermore, our laboratory demonstrated that rice bran modulates intestinal immunity by increasing IgA production (Henderson, Kumar et al., 2012; Yang et al., 2014). In addition, rice varieties have been shown to possess disparate antioxidant potential, suggesting differential ability to neutralize reactive oxygen species generated during acute inflammation (M. H. Chen et al., 2012; Iqbal, Bhanger, & Anwar, 2005; Nam et al., 2006). The implications for differential effects of rice bran from diverse varieties on mucosal immunity and subsequent disease outcomes have not been previously investigated. In the current study we hypothesized that rice bran from diverse rice varieties would differentially modulate mucosal immune-mediated protection against *Salmonella* colonization. Dietary metabolites and intestinal immune responses were associated with protective efficacy against *Salmonella* infection and provide novel evidence for rice bran as a practical weapon against enteric disease.

2. Materials and methods

2.1. *Rice varieties, heat-stabilized rice bran and mouse diet preparation*

Six rice varieties were obtained from the USDA-ARS (Dale Bumpers National Rice Research Center, Stuttgart, AR, USA) as previously described (Forster et al., 2013). The varieties were grown under conventional production practices common to the southern USA rice-growing region. The varieties Red Wells (red bran), IL121-1-1 (red bran), and Jasmine 85 (brown bran) were produced in Beaumont, TX in 2009 while IAC600 (black/purple bran) was produced at the same location in 2007. The varieties Wells (brown bran) and Shufeng 121 (brown bran) were

produced in Stuttgart, AR in 2007 and 2008, respectively. Rough rice of all varieties was stored at 4 °C until 2010 when the samples were prepared for analysis at Beaumont, TX, USA. Samples were dehulled and milled using a Satake One Pass Mill (Pearler, Model SKD, Australia) and the bran was collected and stored at 0 °C for 24 h and shipped on dry ice overnight to Colorado State University. Bran was then heat stabilized in a commercial dryer (Steris, Mentor, OH, USA) at 110 °C for 3 min. Heat stabilized bran was stored at –20 °C and used to prepare mouse diets.

Mouse diets containing 10% (w/w) rice bran were prepared as described previously (Kumar et al., 2012). Briefly, all diets were prepared in house and the AIN-93 maintenance diet (AIN-93) was used as the basal diet (Harlan Teklad, Madison, WI, USA) (Harlan, 2008; Henderson, Kumar et al., 2012; Kumar et al., 2012). Rice bran diet formulation changes were made to casein (reduced to 125 g/kg), corn starch (reduced to 422.7 g/kg), corn oil (reduced to 19 g/kg), and cellulose (reduced to 29 g/kg) to balance macronutrient composition across diets. Mouse diets were vacuum-sealed and stored at 4 °C until use.

2.2. Mice and bacterial infection

Four week old, female 129S6/SvEvTac mice were purchased from Taconic Laboratories (Germantown, NY, USA). Mice were housed under pathogen-free conditions with filtered air. *Salmonella enterica* serovar Typhimurium strain 14028s was a generous gift from Dr. Andres Vazquez-Torres (University of Colorado, Denver, CO, USA). Oral (p.o.) infections were performed as described previously (Kumar et al., 2012). Briefly, glycerol stocks containing 20% (v/v) glycerol were thawed from –80 °C and directly diluted with PBS (Calbiochem, Billerica, MA, USA) to an infectious dose of 2×10^7 CFU in a total volume of 0.2 mL with a 25 GA stainless steel gavage needle. Infectious doses were confirmed by plating each inoculum on MacConkey's agar plates (BD Biosciences, San Jose, CA, USA) plus 50 μg/mL Kanamycin (Fisher Scientific, Pittsburgh, PA, USA) (MAC). The Institutional Animal Care and Use Committee at Colorado State University approved all experiments involving animals.

2.3. Experimental design

All mice were standardized by transferring onto AIN-93 diet from standard chow pellets 7 days prior to initiation of experimental treatments. Following standardization, mice were placed on either a prophylactic or therapeutic rice bran intervention (Supplementary Fig. S1).

For prophylactic investigations three different experimental readouts were performed. Experiment 1 (Exp.1): Mice (n = 10) were transferred to AIN-93 control diet that was modified to include 10% (w/w) of a single rice bran type or were maintained on control diet for seven days. Mice were then infected with *S. enterica* orally as described above, and faecal shedding was measured on days 2, 4, and 6 following infection (Section 2.4). Experiment 2 (Exp. 2): Mice (n = 5–8) were transferred to experimental rice bran diets (10% g w/w) or control diet for seven days. Tissues were harvested and flow cytometry was performed as described below (Section 2.7). Experiment 3 (Exp. 3): Mice (n = 5–8) were fed 10% w/w rice bran diet or control diet for seven days, and then infected with *S. enterica* orally. Seven days after infection mice were euthanized for analysis of immune responses by flow cytometry and histopathology as described below (Sections 2.7 and 2.8).

Experiment 4 (Exp. 4) was performed in a therapeutic design where mice (n = 10) were initially infected with *S. enterica* while consuming the control diet, and then two days after infection mice were transferred onto a 10% rice bran diet for an additional 7 days. On days 2, 4, 6 and 8 after infection body weight and faecal shedding titres were determined (Section 2.4), and on day 9 post infection mice were euthanized. Tissues were processed for flow cytometry as described below (Section 2.7).

2.4. Faecal shedding

Faecal shedding titres were determined as described previously (Kumar et al., 2012). Briefly, faecal pellets were collected from individual mice by transferring each mouse into a separate plastic container. Faecal pellets were suspended in sterile PBS and vortexed for 5 min to solubilize the pellets. Serial 10-fold dilution of faeces homogenate was prepared in sterile PBS and plated on MAC agar plates. Agar plates were incubated at 37 °C for 24 h and CFU/g of faeces were determined from colony counts.

2.5. Analysis and correlation of rice bran fibre fractions and mineral concentrations with faecal shedding

Total dietary fibre, insoluble dietary fibre, and soluble dietary fibre were determined as described previously (Prosky et al., 1994). For mineral analysis, homogenized rice bran samples were oven dried (60 °C), sub-samples were weighed (two per variety), and these were digested using trace metal grade nitric acid (Fisher Scientific) and hydrogen peroxide (GFS Chemicals, Powell, OH, USA) as previously described (Farnham, Keinath, & Grusak, 2011). Elemental analyses were performed on resuspended digests (in 2% nitric acid) using inductively coupled plasma–optical emission spectroscopy (CIROS ICP Model FCE12; Spectro, Kleve, Germany) as described previously (Farnham, Keinath, & Grusak, 2011). Certified rice flour standard (SRM 1568A; National Institute of Standards and Technology, Gaithersburg, MD, USA) was digested and analysed along with the rice bran samples to ensure accuracy of the instrument calibration.

For correlation with faecal shedding titres, fibre percentages and mineral concentrations were determined as described above; in addition, previously reported total fat, fatty acids and total soluble phenolic values were used (Forster et al., 2013). Average faecal shedding titres for each diet across days 2, 4 and 6 were correlated to rice bran nutrient and metabolite concentrations across the six varieties. A two-tailed Spearman's correlation was used to identify metabolites significantly correlated with faecal shedding.

2.6. Tissue digestion

Single cell suspensions were obtained from small intestinal lamina propria cells as described previously (Goodyear, Kumar, Dow, & Ryan, 2014). Briefly, the entire small intestine was harvested and placed in complete media + antibiotics on ice. Next the tissue was flushed with PBS, weighed and cut into 3–4 cm lengths. Mucous was removed by adding 20 mL/g of tissue of

Hank's balanced salt solution (HBSS) (Sigma-Aldrich, St. Louis, MO, USA) with 2% Foetal Bovine Serum (FBS), antibiotics (GI-HBSS), and 5 mM DTT (Dithiothreitol; Amresco, Solon, OH, USA). Tissues were incubated at 37 °C with shaking at 200 rpm for 20 min. Next, epithelial cells were removed in GI-HBSS containing 5 mM EDTA (Ethylenediaminetetraacetic acid; MP Biomedicals, Solon, OH, USA). Three washes were performed at 37 °C with shaking at 200 rpm for 15 min using 15 mL/g of tissue. Next the tissue was digested in GI HBSS with 10 mM HEPES and 0.2 Wünsch units/mL liberase TM (Roche Applied Sciences, Indianapolis, IN) and 200 units/mL DNase (Sigma-Aldrich). Mesenteric lymph node (MesLN) and Peyer's patches (PP) were processed separately, minced into small pieces and digested in HBSS with 10 mM HEPES, antibiotics, 0.1 Wünsch units/mL liberase TM and 200 units/mL DNase. All tissues were triturated through an 18 GA needle, filtered through a 70 micron cell strainer (BD Biosciences), and the cell pellet was washed 2× in HBSS and resuspended in cRPMI.

2.7. Flow cytometry

Flow cytometry was performed as described previously (Goodyear et al., 2014). Briefly, single cell suspensions were prepared as described above, and blocked with FACS block for 5 min prior to antibody incubation. Next, surface antibodies were added and incubated at 4 °C for 30 min. Surface antibody clones and fluorescent conjugates used in this study were the same as those described previously (Goodyear et al., 2014). Following incubation with surface antibodies cells were washed with FACS buffer and incubated with streptavidin pacific orange (Life Technologies, Grand Island, NY, USA) for 20 min at 4 °C. Cells were then fixed in 1% Paraformaldehyde (PFA), washed in FACS, and resuspended in FACS until analysis.

Flow cytometry was performed using a Gallios flow cytometer using Gallios software version 1.2 (Beckman Coulter, Fullerton, CA, USA). Analysis was performed using FlowJo software version 7.6.5 (Tree Star Inc., Ashland, OR, USA). Cell populations were defined as follows: neutrophils (PMN): $CD45^+/Siglec\text{-}F^-/CD11b^+/Ly6G^+$, monocytes: $CD45^+/Siglec\text{-}F^-/CD11b^+/Ly6G^-/Ly6C^+$, macrophage: $CD45^+/Siglec\text{-}F^-/CD11b^+/Ly6G^-/Ly6C^-/F480^+$, interdigitating dendritic cells (iDC): $CD45^+/Siglec\text{-}F^-/CD11c^+/MHC\text{-}II^+/CD103^+$, myeloid dendritic cells (mDC): $CD45^+/Siglec\text{-}F^-/CD11c^+/MHC\text{-}II^+/CD11b^+$, plasmacytoid dendritic cells (pDC): $CD45^+/Siglec\text{-}F^-/CD11c^+/MHC\text{-}II^+/PDCA^+$, $CD8^+$ T cells: $FSC\text{-}SSC^{low}/DX5^-/CD3^+/CD8^+$, $CD4^+$ T cells: $FSC\text{-}SSC^{low}/DX5^-/CD3^+/CD4^+$, γδ T cells: $FSC\text{-}SSC^{low}/CD45^+/\gamma\delta\,TCR^+$, regulatory T cells ($T_{reg}$): $FSC\text{-}SSC^{low}/DX5^-/CD3^+/FoxP3^+$, natural killer (NK) cells: $FSC\text{-}SSC^{low}/CD3^-/DX5^+$, B cells: $FSC\text{-}SSC^{low}/CD45^+/CD3^-/DX5^-/B220^+$, plasma cells: $FSC\text{-}SSC^{low}/CD45^+/CD138^+$, and eosinophils: $CD45^+/Siglec\text{-}F^+$.

2.8. Histological analysis

Fixation and processing of tissues for histopathology analysis were performed as described previously (Goodyear, Bielefeldt-Ohmann, Schweizer, & Dow, 2012). Briefly, following euthanasia, the distal 10 cm of the small intestine was collected and arranged in a "swiss roll" configuration. Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich), embedded in paraffin and sectioned. Tissue sections were then stained with haematoxylin and eosin and evaluated by a board certified veterinary pathologist (E.J. Ehrhart). All the tissues were evaluated blind to treatment group and scored 0–3 for severity (0 = not present, 1 = mild, 2 = moderate, 3 = marked) as compared to normal age matched mouse intestine for the following criteria: neutrophils, mononuclear inflammatory cells, mucosal surface epithelial necrosis, luminal surface pseudomembrane formation and presence of serositis (suggesting transmural inflammation). Each of these criteria was scored for the intestine overall as well as for areas specific to PP.

2.9. Statistical analysis

Statistical analysis was performed with Graph Pad Prism version 5 software (Graph Pad, San Diego, CA, USA). Correlation analysis was performed by a two-tailed Spearman analysis. Analysis between two groups was performed by a two-tailed Mann–Whitney test. Comparisons between three or more groups were performed by a Kruskal–Wallis one-way ANOVA followed by a Dunn's multiple means comparison. For comparisons between multiple groups over time a two-way ANOVA followed by a Bonferroni post-test was used. Differences were considered statistically significant for $p < 0.05$, and trends were considered at $p < 0.1$.

3. Results

3.1. Rice bran varieties have different protective efficacies against Salmonella colonization

To investigate potential differences between rice varieties, mice were prophylactically fed rice bran and infected with *S. enterica* as described in Exp. 1 in Materials and Methods. Both varietal and temporal differences in faecal shedding were observed (Fig. 1). For instance, IAC600 consistently reduced *Salmonella* faecal shedding throughout the course of the experiment ($p < 0.05$). In contrast, beneficial effects of IL-121-1-1 and Red Wells were transient, with reduction in faecal shedding observed at day 4 ($p < 0.05$) (Fig. 1). Differences in efficacy were observed between diets containing IAC600 and Shufeng 121 on day 2 ($p < 0.05$), and trends were observed between Red Wells and Wells on day 4 and between IAC600 and Shufeng 121 on day 6 ($p < 0.10$). *Salmonella* faecal shedding was not different between rice bran diets containing Wells, Jasmine 85 or Shufeng 121 as compared to control diet.

3.2. Differential fibre, fatty acid, vitamin and mineral concentrations correlate with protective efficacy

To identify potential rice bran bioactive components responsible for differential protection, the fibre proportions and mineral concentrations across varieties were determined (Table 1, Supplementary Table S1). Next, correlations between nutrient concentrations and faecal shedding titres were identified as described in the methods. Spearman's analysis revealed significant negative correlations ($p < 0.05$) between *Salmonella* faecal shedding and total organic matter ($r = -0.94$), soluble fibre ($r = -0.80$), α-tocopherols ($r = -0.89$), stearic acid (18:0) ($r = -0.94$),

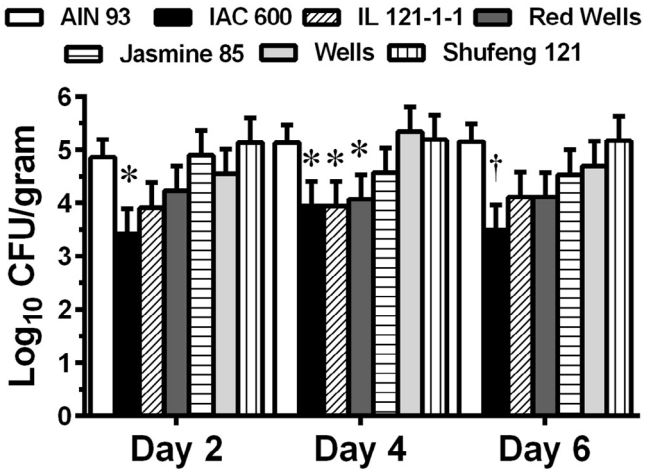

Fig. 1 – Effect of dietary rice bran of different varieties on *Salmonella* faecal shedding. Consumption of IAC600, IL 121-1-1 and Red Wells rice bran significantly reduced faecal shedding in mice while Jasmine 85, Wells and Shufeng 121 rice bran diets did not. 129SvEvTac mice were fed AIN-93 diet for 7 days and then either remained on AIN-93 diet (control diet) (n = 30) or were transferred onto AIN-93 diet containing 10% rice bran (n = 10 per diet group). Mice were fed with rice bran from a diverse panel of varieties and faecal shedding (CFU/g of faeces) was measured 2, 4 and 6 days following oral infection with 2×10^7 CFU *S. enterica* 14028s. Statistical differences were determined by a repeated measures two-way ANOVA followed by a Bonferroni post-test. Data were pooled from three independent experiments. *$p < 0.05$, †$p < 0.01$. Data are presented as mean $\log_{10}$ CFU/g ± SEM.

lignoceric acid (24:0) (r = –0.94), and boron (r = –0.89). Negative trends ($p < 0.10$) were observed with gallic acid equivalents (r = –0.83) and behenic acid (22:0) (r = –0.81). Positive correlations were observed with γ-tocotrienol ($p < 0.05$; r = 0.94), as well a trend ($p < 0.10$) for α-linoleic acid (18:3n3) (r = 0.84).

3.3. *Differential intestinal immune response elicited by rice bran varieties*

Differences between *Salmonella* protection and rice bran metabolites led us to investigate immune responses associated with these rice varieties. Global intestinal immune response analysis was focused on a subset of rice varieties including IAC600, Red Wells and Wells. IAC600 was selected because it was consistently the most effective rice variety with regard to preventing *Salmonella* colonization. Red Wells and Wells were chosen as diets that were moderately effective (Red Wells) and ineffective (Wells) for protecting against *Salmonella* infection, despite high levels of genetic similarity (Brooks, Yan, Jackson, & Deren, 2008; Forster et al., 2013; Ryan et al., 2011). Mice were treated as described in Exp. 2 in the Materials and Methods section and cellular immune responses were investigated.

Immune analysis revealed differences in responses to rice varieties, as well as intestinal tissue compartmentalization (Fig. 2). For example, IAC600 diet had a dramatic effect on the small intestine (SI) immune response increasing multiple cell types when compared to AIN-93 diet including macrophages, iDCs, $CD8^+$ T cells, γδ T cells, and T_{reg} ($p < 0.05$). In addition, IAC600 resulted in increased PMN, macrophages, mDCs, $CD8^+$ T cells, γδ T cells, and plasma cells when compared to Red Wells or Wells diets ($p < 0.05$). Intestinal immune responses to Red Wells and Wells were less pronounced with Wells diet resulting in increased pDC over control diet, while monocytes and $CD4^+$ T cells increased after feeding Red Wells and Wells containing diet relative to diet containing IAC600 bran. In contrast to SI tissue, no differences in immune populations were observed in Peyer's Patches (PP) regardless of diet, while Red Wells diet induced increases in $CD8^+$ and γδ T cells in the mesenteric lymph nodes (MesLN) ($p < 0.05$).

Table 1 – Fibre fractions of bran for the six rice varieties tested.[a]

Rice variety	DM	OM	Dietary fibre		
			Total	Insoluble	Soluble
IAC600	88.42	94.01	25.21	23.66	1.55
Red Wells	87.58	91.93	22.19	21.53	0.67
Jasmine 85	86.87	92.83	16.20	16.76	0.00
IL 121-1-1	88.56	91.51	25.66	29.75	0.00
Shufeng 121	89.79	91.03	27.75	29.64	0.00
Wells	89.92	90.88	24.11	24.50	0.00

DM = Dry matter; OM = Organic matter.
[a] All values are expressed as a per cent of dry matter.

3.4. *Intestinal immune responses persist despite* Salmonella *infection*

Tissue histopathology was utilized to investigate the effects of prophylactic rice bran consumption on *Salmonella* infection treated as described in Exp. 3. The histological scoring was analysed to focus on three rice varieties for which immune profiling was performed. The inflammatory response following infection of control fed mice was multifocal, and the PP involved heavy neutrophilic inflammation with admixed necrosis, as well as protein exudation and loss of lymphocytes. Segments of the intestine without a PP uniformly had more modest changes that included an infiltration of the lamina propria by neutrophils. Although no significant differences in histopathology scores were observed between rice varieties, qualitative observations revealed differential inflammatory responses. Fig. 3 shows that the PP in mice consuming IAC600 diet had reduced necrosuppurative inflammation, oedema and fibrin leakage, improved vascular integrity and reduced luminal pseudomembrane formation as compared to the AIN-93 or Wells diet. The lymphoid follicles of PP in AIN-93 and Wells small intestines were severely lymphocyte-depleted and partially effaced by the necrosuppurative inflammation. Neutrophils, when present in IAC600 fed mice, were intact and nondegenerate as compared to those in AIN-93 and Wells intestine, which had degenerate karyorrhectic neutrophils embedded within the necrosis and fibrin exudation. The intestines of mice fed Red Wells diet had intermediate changes compared to IAC600 and Wells diets.

To more specifically investigate global immune changes in response to rice bran consumption and *Salmonella* infection,

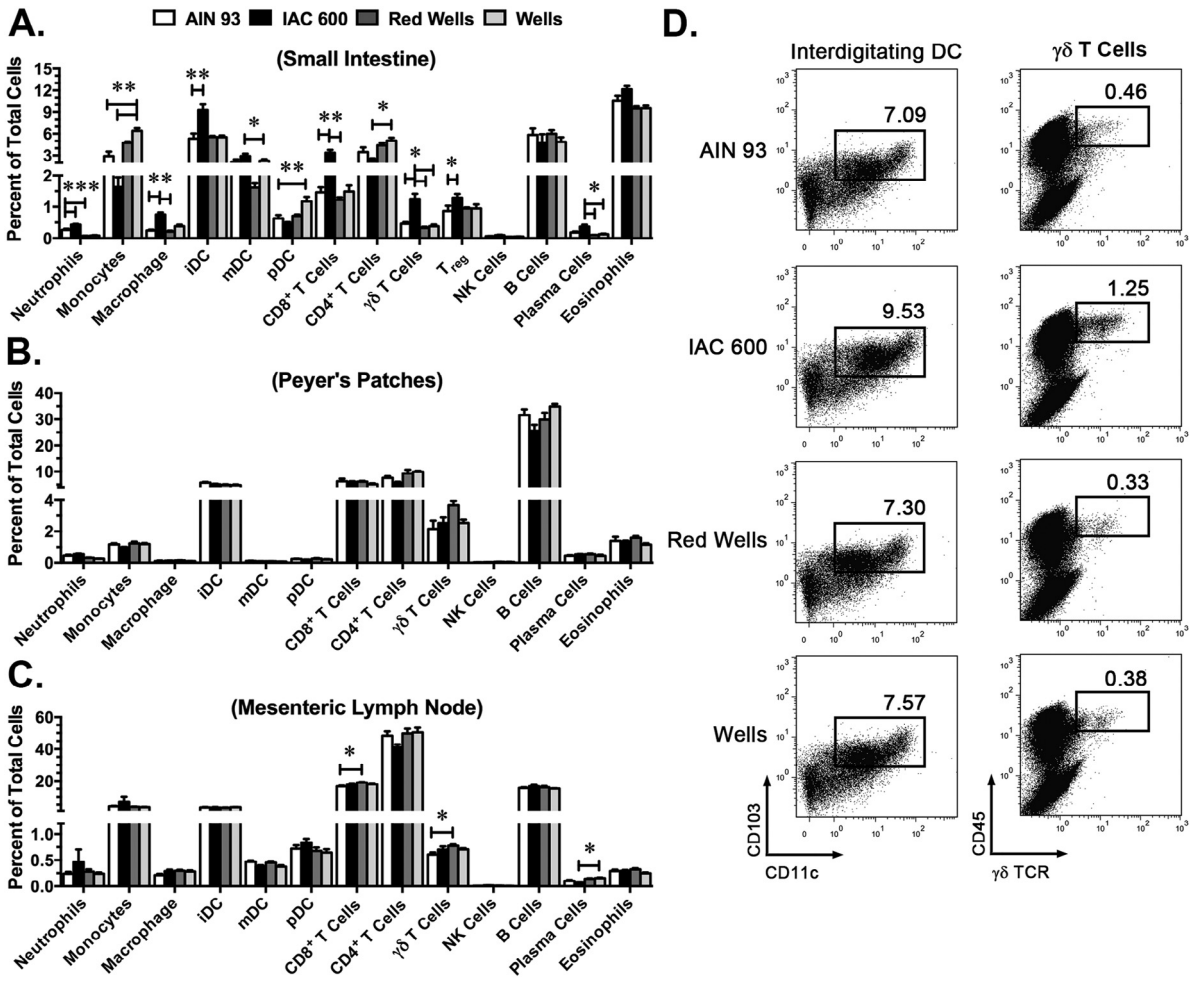

Fig. 2 – Rice bran from different varieties elicit differential intestinal immune responses. SI changes in neutrophils, antigen presenting cells, T cells and B cells were observed, while minimal changes in PP or MesLN populations were observed. 129SvEvTac (n = 5–8) mice were placed on AIN-93 diet for 7 days and then remained on AIN-93 diet or were transferred onto AIN-93 diet containing 10% rice bran. Seven days after transferring onto rice bran diet mice were euthanized, single cell suspensions were prepared and analysed by flow cytometry as described in materials and methods section. Graphical representation of innate and adaptive cell populations from small intestine (A), Peyer's patches (B) or mesenteric lymph node (C) are presented as mean ± SEM. Statistical differences were determined by a Kruskal–Wallis one-way ANOVA followed by a Dunn's multiple means comparison. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Representative dot plots of interdigitating DCs and γδ T cells from the small intestine are presented in (D). Dot plots were generated by pooling all samples from each group in Flow Jo software and the percentage of total cells is reported on each dot plot. Preliminary gating strategies for representative dot plots include: interdigitating DCs: $CD45^+/SiglecF^-/Ly6G^-/MHC\text{-}II^+$; γδ T cells: $FSC\text{-}SSC^{low}$. Data are representative of two independent experiments. iDC = interdigitating dendritic cells; mDC = myeloid DC; pDC = plasmacytoid DC.

flow cytometry was performed with a focus on the most effective variety (IAC600). Similar to the effects of rice bran alone, *S. enterica* infection of mice fed IAC600 diet induced increases in $CD8^+$ and γδ T cells ($p < 0.05$) (Fig. 4), and trends towards increased iDCs were also observed ($p < 0.10$). In contrast, a reduction in pDCs was observed in mice fed the IAC600 diet. *Salmonella* infection following IAC600 diet also resulted in differences in PP immune populations, including trends towards increased PMNs and γδ T cells in IAC600 fed mice ($p < 0.10$), and a decrease in macrophages ($p < 0.05$). However, no differences in MesLN populations were induced in response to IAC600 consumption and *S. enterica* infection.

Further evidence supporting the ability of IAC600 rice bran to modulate inflammatory responses, despite *S. enterica* infection, was obtained in preliminary therapeutic studies with IAC600 as described in Exp. 4 within the Materials and Methods section. Consumption of IAC600 rice bran resulted in significant reductions in faecal shedding (6.2 ± 0.3 vs 4.9 ± 0.3 $\log_{10}$ CFU/g; $p < 0.05$, two-way ANOVA) as well as increased body weight compared to mice fed AIN-93 diet (93.9 ± 4.6 vs 105.4 ± 1.6 per cent; $p < 0.05$, two-way ANOVA) (Supplementary Fig. S2). Furthermore, immune responses investigated 8 days post-infection revealed mice consuming IAC600 had significant reductions in intestinal PMN recruitment and increases in natural killer (NK)

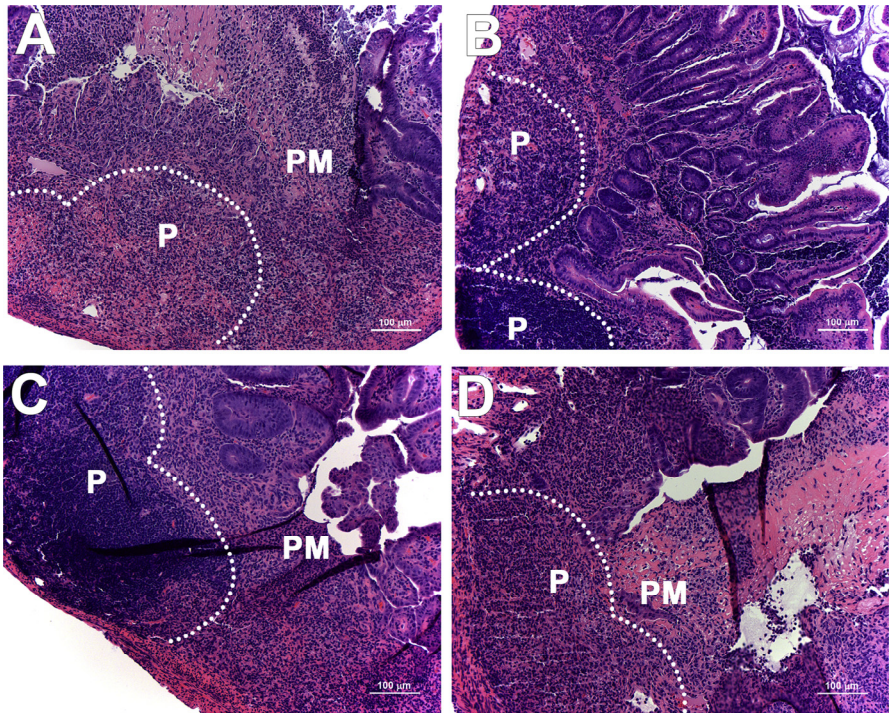

Fig. 3 – Reduced inflammation in Peyer's patches reflects changes in *Salmonella* faecal shedding. 129SvEvTac (n = 5) mice were placed on AIN-93 diet for 7 days and then remained on AIN-93 diet (A) or were transferred onto AIN-93 diet containing 10% of a rice bran variety, 10% IAC600 rice bran (B), 10% Red Wells rice bran (C), or 10% Wells rice bran (D). Mice were then infected with 2×10^7 CFU *S. enterica* 14028s orally and were maintained on control or rice bran diets for seven days, tissues were fixed in 10% formalin, sectioned and stained with H + E as described in methods. Representative images are of the intestine with PP. Mice on Wells diet had similar changes to mice on the basal AIN-93 diet that included lymphoid depletion of the Peyer's patches (P) and effacement by necrosuppurative inflammation with epithelial necrosis and luminal pseudomembrane (PM) formation. Mice fed IAC600 and Red Wells diets had maintained integrity of the PP with variable reduction in necrosis.

cells and eosinophils, while trends towards increased T_{reg} were observed. Changes in PP included reduced mDC and $CD8^+$ T cells, as well as increased B cells. Similar to SI tissue, increases in NK cells and eosinophils were observed in MesLN (Supplementary Fig. S2). Finally, upon normalization to bacterial burden, decreases in PMNs and mDCs observed in raw data were no longer evident while increases in iDC, γδ T cells, NK cells, and eosinophils were observed across SI and MesLN tissues ($p < 0.05$, two tailed Mann–Whitney test) (data not shown).

4. Discussion

In the present study, we demonstrate differential capacity of bran from rice varieties to reduce *Salmonella* colonization in mice (Fig. 1). Metabolite analysis revealed that reduced faecal shedding was correlated with increased concentrations of soluble fibre, long chain fatty acids (stearic acid, lignoceric acid), vitamin E (α-tocopherol) and minerals (boron) in rice bran varieties. Rice bran consumption induced different immune responses, depending on the variety. IAC600 was the most beneficial variety for inhibition of *Salmonella* and altered multiple cell populations including iDC, $CD8^+$ T cells, γδ T cells and T_{reg}, while diets of Red Wells or Wells, which conferred intermediate or no protection, respectively, had reduced capacity to modulate intestinal immunity. The majority of changes were observed in intestinal tissue, although increased $CD8^+$ T cells and γδ T cells were observed in the MesLN of mice consuming Red Wells, suggesting certain diets may induce migration of immune cells to lymphatic tissues. In addition, similar results were obtained in preliminary studies investigating therapeutic rice bran treatment, further demonstrating the capacity of rice bran to positively influence enteric disease outcomes.

Following Neptune rice bran consumption, reduced tissue damage and circulating TNF-α, IL-12p40, and IFN-γ were observed, despite similar lymphocyte numbers, suggesting that suppression of inflammation may be an important mechanism of action for rice bran protection against *Salmonella* (Kumar et al., 2012). Focusing on the most protective variety, IAC600, differences in SI macrophages, iDC and T_{regs}, were observed exclusively with this variety (Fig. 2). iDCs have been shown to induce regulatory T cells and thus these cells, in the presence of rice bran, may be driving an anti-inflammatory environment (Flores-Langarica et al., 2012). Furthermore, recent studies have highlighted the importance of immunosuppressive T_{reg} in response to *Salmonella* infection, potentially through suppression of acute pro-inflammatory innate immune

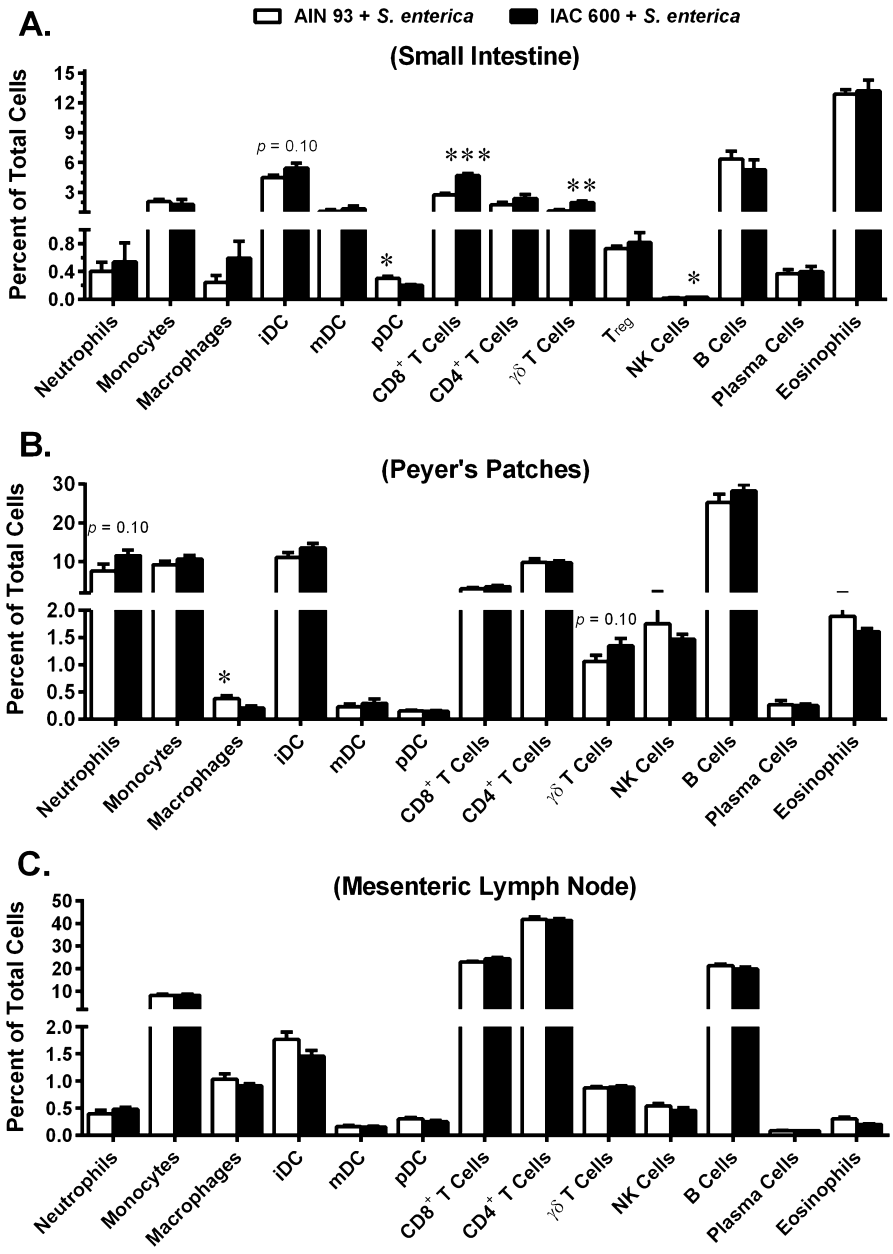

Fig. 4 – Rice bran consumption maintains intestinal immune response after *S. enterica* infection. 129SvEvTac (n = 8) mice were placed on AIN-93 diet for 7 days and then remained on AIN-93 diet or were transferred onto AIN-93 diet containing 10% rice bran. Mice were then infected with 2×10^7 CFU *S. enterica* 14028s orally and were maintained on control or rice bran diets. Seven days after infection mice were euthanized, single cell suspensions were prepared and analysed by flow cytometry as described in the materials and methods section. The percentage of total cells is presented for small intestine (A), Peyer's patches (B) or mesenteric lymph node (C) as mean ± SEM. Statistical differences were determined by a two-tailed Mann–Whitney test. *p < 0.05, **p < 0.01, ***p < 0.001. Data are representative of two independent experiments.

responses (Broz, Ohlson, & Monack, 2012; O'Mahony et al., 2008; Scully et al., 2013). *Salmonella* is known to promote pro-inflammatory responses that are necessary for colonization (Stecher et al., 2007). Therefore, based on histopathology and flow cytometry results of IAC600 rice bran, suppression of intestinal inflammation and expansion of T_{reg} cells may be important for protection against *Salmonella*-induced enteritis.

Soluble fibre components of the bran demonstrated importance in the current study as the soluble fraction correlated with protection against *Salmonella* (Table 2). Soluble fibre modulates immunity by reducing inflammatory cytokine expression by mouse macrophages, including TNF-α, IL-1β, IL-12 and IFN-γ following *ex-vivo* lipopolysaccharide (LPS) challenge (Sherry et al., 2010). Gut microbes are known to process soluble fibre into short chain fatty acids (SCFA), which have multiple beneficial effects,

Table 2 – Correlation of rice bran metabolites and *Salmonella* faecal shedding.[a]

Component	r	p-value
Fibre		
Organic matter	–0.94	0.02*
Total fibre	0.31	0.56
Soluble fibre	–0.8	0.03*
Insoluble fibre	0.54	0.3
Phenolics		
GAE	–0.83	0.06§
Antioxidants		
γ-oryzanol	0.09	0.92
Vitamin E[b]		
α-tocopherols	–0.89	0.03*
γ-tocopherols	0.6	0.24
δ-tocopherols	0.4	0.52
α-tocotrienol	–0.54	0.3
γ-tocotrienol	0.94	0.02*
δ-tocotrienol	–0.54	0.3
Total vitamin E	0.14	0.8
Fatty acids[b]		
Myristic acid (14:0)	–0.41	0.42
Palmitic acid (16:0)	0.49	0.36
Palmitoleic acid (16:1)	0.06	0.92
Stearic acid (18:0)	–0.94	0.02*
Oleic acid (18:1n9)	0.03	1.0
Vaccenic acid (18:1 n7)	0.37	0.5
Linoleic acid (18:2 n6)	–0.14	0.8
α-linolenic acid (18:3n3)	0.84	0.06§
Arachidonic acid (20:0)	–0.03	1.0
Gadoleic acid (20:1)	0.37	0.5
Behenic acid (22:0)	–0.81	0.06§
Lignoceric acid (24:0)	–0.94	0.02*
Elements		
Boron	–0.89	0.03*
Calcium	–0.6	0.24
Cobalt	0.43	0.42
Copper	–0.03	1.0
Iron	–0.09	0.92
Potassium	0.2	0.71
Magnesium	0.26	0.66
Manganese	0.43	0.42
Molybdenum	–0.26	0.66
Nickel	–0.77	0.1
Phosphorus	–0.09	0.92
Sulphur	–0.43	0.42
Zinc	–0.54	0.3

[a] Correlation of mean $\log_{10}$ CFU/g *Salmonella* faecal shedding (days 2, 4 and 6; Fig. 1) with mean values from metabolite analysis as determined by a two-tailed Spearman analysis.

[b] Correlations performed with previously reported metabolite levels (Forster et al., 2013).

* $p < 0.05$;

§ $p < 0.1$.

r = Spearman r value; GAE = Gallic acid equivalents.

Vitamin E levels: μg/gram of rice bran; γ-oryzanol = mg/gram of rice bran; GAE = ng/mg of methanol extract; Fatty acids and fibre = Per cent of rice bran; Ca, P, Mg, P, S = mg/gram rice bran; As, Bo, Co, Cu, Fe, Mn, Mo, Ni, Zn = μg/gram rice bran.

including an altered intestinal pH, increased epithelial cell integrity, and immune modulation inducing T_{reg} expansion, induction of antimicrobial peptides, cytokine and chemokine production, and altered cell migration (Arpaia et al., 2013; Harrison, Balan, & Babu, 2013; Kaczmarczyk, Miller, & Freund, 2012; Vinolo, Rodrigues, Nachbar, & Curi, 2011). Moreover, studies have demonstrated the ability of SCFA to mitigate *Salmonella* infections, including reduced faecal shedding and decreased expression of bacterial invasion genes (Harrison et al., 2013). Our recent publication demonstrated that dietary rice bran increases native gut *Lactobacillus* spp., which are also known to protect mice against *Salmonella* infection (Asahara et al., 2011; Kumar et al., 2012). In addition to SCFA production, microflora metabolism may also be important for release of bound phenolics (Russell, Labat, Scobbie, & Duncan, 2007). Bound phenolics are known to be important in other diseases such as type 2 diabetes (Belobrajdic & Bird, 2013). A trend towards correlation with increased total phenolics was observed in the current study (Table 2), and as such specific investigation of bound phenolics is warranted. Further studies will be needed to determine how microflora metabolism of rice bran, SCFAs, and/or bound phenolics of rice bran varieties differentially influences *Salmonella* colonization.

Further evidence supporting the importance of the microflora includes recent studies demonstrating that a bioprocessed polysaccharide (BPP) derived from fermentation of black rice bran by *Lentinus edodes* can protect against systemic *Salmonella* infection and LPS mediated shock (Kim et al., 2013, 2014). Following systemic *Salmonella* infection, BPP was found to activate macrophages *in-vitro* and induced splenic production of IL-12 and IFN-γ, but not IL-10 (Kim et al., 2014). Similar to the black rice bran used to develop BPP, we identified a pigmented rice variety, IAC600, as the most protective variety against enteric *Salmonella* infection. The current study expands upon BPP findings to show that IAC600 rice bran can modulate intestinal immune responses such as T_{reg}, as well as $CD8^+$ and γδ T cells, DC and macrophages (Fig. 2). One potential limitation of the current study is the use of bran from genetically diverse rice varieties that were grown under different environmental conditions. While there is some evidence for genetics and environment (GxE) to affect the levels of certain rice bran components (Bergman & Xu, 2003; Miller & Engel, 2006), most studies use commercially sourced rice bran and do not provide documentation of growing environment. As such, the concentration ranges for vitamin E isomers, gamma-oryzanol, boron, and other compounds could influence the correlations observed herein with bioactivity, and these differences cannot be conclusively a result of variety or environment. Moreover, agronomic conditions may affect the relative importance of pro- and anti-inflammatory immune responses associated with reductions in *Salmonella* colonization in mice fed the IAC600 rice bran variety. Therefore, we cannot exclude the possibility that the rice production environment may contribute to differential protective effects, and is another variable that merits exploration in future studies.

In summary, the current study highlights differential capacity of bran from rice varieties to inhibit *Salmonella* colonization and to modulate intestinal immunity. Analysis of bran metabolites revealed multiple potential protective mechanisms including immunomodulation and intestinal microbiome modulation. Further support for rice bran as a functional food comes from recent studies where rice bran dietary supplementation to milk prevented diarrhoea in a gnotobiotic rotavirus infected neonatal pig, and rice bran offered additional protection when co-administered with vaccine (Yang et al., 2014).

5. Conclusions

Altogether these studies highlight the capacity of rice bran to combat enteric disease and support the need for an improved understanding of the complex interactions between dietary interventions, intestinal immunity, and microflora in the context of protection against enteric pathogens. Further investigation of $CD4^+$ T cell subsets, γδ T cell subsets, innate lymphoid cells (ILC) and the role NK cells may be playing in protection is needed, as well as further experiments expanding upon preliminary findings that rice bran may have use as a therapeutic intervention for enteric diseases.

Conflict of interest

The authors disclose no conflicts of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.08.027.

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